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RESEARCH ARTICLE

REVISED **Lack of change in CA1 dendritic spine density or clustering in rats following training on a radial-arm maze task**
[version 2; peer review: 2 approved]

Emma Craig, Christopher M. Dillingham, Michal M. Milczarek, Heather M. Phillips, Moira Davies , James C. Perry *, Seralynne D. Vann *

School of Psychology, Cardiff University, Cardiff, CF10 3AT, UK

* Equal contributors

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Abstract

Background: Neuronal plasticity is thought to underlie learning and memory formation. The density of dendritic spines in the CA1 region of the hippocampus has been repeatedly linked to mnemonic processes. Both the number and spatial location of the spines, in terms of proximity to nearest neighbour, have been implicated in memory formation. To examine how spatial training impacts synaptic structure in the hippocampus, Lister-Hooded rats were trained on a hippocampal-dependent spatial task in the radial-arm maze.

Methods: One group of rats were trained on a hippocampal-dependent spatial task in the radial arm maze. Two further control groups were included: a yoked group which received the same sensorimotor stimulation in the radial-maze but without a memory load, and home-cage controls. At the end of behavioural training, the brains underwent Golgi staining. Spines on CA1 pyramidal neuron dendrites were imaged and quantitatively assessed to provide measures of density and distance from nearest neighbour.

Results: There was no difference across behavioural groups either in terms of spine density or in the clustering of dendritic spines.

Conclusions: Spatial learning is not always accompanied by changes in either the density or clustering of dendritic spines on the basal arbour of CA1 pyramidal neurons when assessed using Golgi imaging.

Keywords

Golgi stain, hippocampus, spatial memory, spinogenesis

Open Peer Review

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1 **Michael G. Stewart** , The Open University, Milton Keynes, UK

2 **Bruce Harland** , University of Auckland, Auckland, New Zealand

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Seralynne D. Vann (vannsd@cardiff.ac.uk)

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REVISED Amendments from Version 1

Method – Image Analysis section. We have clarified that segments needed to be fully impregnated by Golgi stain to be included for analysis.

Method – Image analysis section. We have clarified that branched spines were only counted as one spine.

Method – Image analysis section. We have clarified how our image processing method ensured spines deeper in the tissue section were not missed from being counted.

Method – Image analysis section. We have clarified that a 0.2µm step size was used to collect image stacks.

Method – Image analysis section. We have clarified that spines above or below the dendrite with respect to the field of view are not visible with Golgi stain and hence no attempt to count or correct for them was made.

Method – Golgi staining. We have now clarified that sections were sliced in the coronal plane.

Method – Image analysis. We have clarified that CA1 basal dendritic segments were imaged from the dorsal region of CA1 extending -2.7mm to -4.6mm posterior to bregma according to the atlas of Paxinos and Watson (2006).

Discussion – We have added a section discussing spine changes in CA1 basal dendrites and apical dendrites following spatial memory training.

Discussion – We have added a section discussing why spines were not classified into discrete categories and have suggested an approach for future studies.

References – Additional references added to reference list.

Any further responses from the reviewers can be found at the end of the article

Introduction

The hippocampus plays a vital role in spatial learning and memory^{1,2}. Since the discovery of place cells in the Cornu Ammonis 1 (CA1) field of the hippocampus³, this subregion has become a major focus of research into spatial memory. Numerous studies, across species, have identified a role for CA1 in spatial learning and memory. For example, rats with CA1 lesions are impaired on spatial memory tasks⁴. Furthermore, the extent of pyramidal neuron loss within CA1 has been found to correlate with performance on a T-maze task, regardless of overall hippocampal damage⁵. This is consistent with findings from patient studies where extent of damage to CA1 correlates with impairment on a virtual place learning task⁶.

Learning and memory is supported by neural plasticity, whereby learning episodes elicit subcellular morphological changes, facilitating the long-term representation of the event. Neural plasticity includes the experience-dependent modification of dendritic spines. Excitatory neuronal firing can increase numbers of CA1 dendritic spines both *in vivo* and *in vitro*^{7,8}. A number of behavioural tasks have been found to bring about an increase in CA1 spine density, with changes most pronounced on the basal arbors of CA1 pyramidal neurons⁹. While there is a long-standing association between memory and spine density, more recent studies have also highlighted the importance not only of the overall number but also the location of the spines. Neighbouring synapses will result in

greater depolarisation of the neuron when simultaneously activated, thus providing a mechanism for greater processing capacity¹⁰. Consistent with this idea, learning has been shown to result in dendritic spines that are located in close proximity, i.e. clustered.

To date, most studies have focused on spine clustering in cortex¹¹, although there is evidence that watermaze training also affects spine clustering in CA1³. However, tasks carried out in the watermaze are typically aversive, so changes can be difficult to interpret as stress has also been shown to affect spine density^{12–14}. Furthermore, it is difficult to identify suitable behavioural controls for watermaze tasks¹⁵. We therefore assessed spine density and clustering in rats that had been trained on appetitive tasks, i.e. working memory version of the radial-arm maze task. This task, and species, was chosen as CA1 activity has been associated with performance on this task^{16–18} and previous studies in rats have reported increased spine density following radial-arm maze training^{19–21}. Mahmoud *et al.*²¹ also found a significant correlation between spine density of CA1 basal arbors and errors on the radial-arm maze task. These results not only suggest that spine density increases following training on the radial-arm maze but that it is also directly linked to performance. Following standard training on the radial-arm maze task, animals underwent further testing where the maze was rotated mid-trial, to ensure animals were performing the task using extramaze cues. Two control groups were included, one behavioural control group that was trained to run up and down one arm of the maze. Behavioural control and experimental animals were, therefore, matched for sensorimotor stimulation and rewards received but differed in terms of mnemonic demand. A further home-cage control group was included, which comprised animals that were age-matched but had undergone no behavioural training.

The current study tested the hypothesis that spatial memory training results in increased spine density and clustering in the CA1 subregion of the hippocampus. As such, we would expect to see differences between the spatial memory group and both of the control groups.

Methods

Animals

Thirty naïve adult male Lister-Hooded rats (Harlan, UK) were involved in the study. The rats were approximately 3 months of age at the start of the experiment and maintained around 300g for the extent of the experiment (approximately 5 weeks). Rats were housed in pairs under diurnal light conditions (14 h light/10 h dark) and any testing was carried out at a regular time during the light phase. Cages were plastic-based with metal bars forming the lid. Sawdust covered the floor of the cage and a cardboard tube was placed within each cage. During the behavioural testing period, animals were food deprived but their body weight did not fall below 85% of free feeding weight. Animals were given access to water throughout. Animals were habituated to handling before commencing the study. The experiment was carried out in accordance with UK Animals (Scientific Procedures)

Act, 1986 and associated guidelines. All efforts were made to ameliorate any suffering of animals. We used an appetitive behavioural task, rather than an aversive task, to minimise stress.

Sample size. Thirty animals were used in total, ten in each experimental condition. This number was arrived at on the basis of previously published studies using similar approaches and addressing similar questions^{19–22}.

Animal allocation. There were three experimental groups: a home-cage group that was food restricted but did not undergo behavioural training; a spatial memory group that was trained on a working memory version of the radial-arm maze task; finally, a yoked-control group were matched for the sensorimotor aspects of radial-arm maze testing but without the memory load by simply running up and down one arm of the maze for food rewards. The animals were allocated randomly to the experimental groups at the outset of the experiment on the basis of rat number so for every three rats there would be one animal in each experimental condition. The only constraining factor was that the home-cage controls were housed together while the other cages contained one yoked control and one spatial memory rat. The spatial memory and yoked control animals were interleaved for behavioural testing. Once the tissue had been processed all slides were anonymised such that all data collection and analysis was carried out with the experimenter blind to experimental group.

Radial-arm maze task

Apparatus. The radial-arm maze consisted of an octagonal central platform with eight equally spaced arms radiating from the central platform. Food wells were located at the end of each arm. The floors of the maze were made of wood and painted white while the walls were made of transparent Perspex. Each arm had a Perspex sliding door, attached to a pulley system, enabling the experimenter to control access to and from the central platform. The entire maze was placed on wheels so that it could be easily rotated. Geometric shapes and other high contrast stimuli were located on the walls.

Behavioural procedure. Rats in the spatial memory group and the yoked controls were brought from the holding room to the testing room in pairs in an opaque carrier case. Rats underwent four habituation sessions where they could freely explore the maze for 10 minutes, for the first two days with all the doors raised and for the second two days with the doors opened and closed. For the first habituation session, rats were placed in the maze in pairs; for the remaining three habituation sessions they were habituated individually. For all habituation sessions, sucrose reward pellets (45mg, LabDiet, St Louis, Missouri, US) were scattered down the arms.

In the training phase for the spatial memory group, all eight arms were baited with a single reward pellet. The rat was placed on the central platform, with all doors closed. The experimenter then opened all the doors allowing the animal to choose an arm to enter. After eating the reward pellet the animal returned to the central platform and the doors

were closed for 10 seconds before being opened again, allowing the rat to make another choice. This continued until all arms were visited or until a 10-minute time limit was reached. The optimal strategy involves retrieving all reward pellets from all 8 arms without entering previously entered arms. An error was scored if a rat entered any arm more than once. Once animals had learnt the standard task, after 12 sessions, a rotation stage was included to ensure animals were using spatial cues to perform the task. The first part of the testing session was identical to the standard version of the task. However, after four correct choices were made, the animal was removed from the maze and the maze was rotated 45 degrees. This was either clockwise or anti clockwise on alternate days. The remaining food pellets were moved so that they were in the same position in relation to the extra-maze cues. Following this, the doors of the maze were re-opened and the animal was allowed to complete the trial, i.e. retrieve the remaining four rewards. After this there was a test phase in which the rat was returned to the central platform until the remaining four reward pellets were retrieved. The animals received six rotation sessions.

Yoked animals spent the same overall amount of time in the radial-arm maze as their counterparts, and received the same number of rewards, but they only had access to one arm of the maze, which remained the same throughout training.

Golgi staining

Ninety minutes after the behavioural animals completed the final test session, they were anaesthetised with sodium pentobarbital and transcardially perfused with 0.1 M phosphate buffer saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS (PFA).

Golgi staining was carried out using the FG Rapid GolgiStain Kit based on the Golgi-Cox impregnation technique. For this, the brains were rinsed in distilled water before being immersed whole in a solution containing mercuric chloride, potassium dichromate and potassium chromate (kit solutions A+B) and stored in darkness at room temperature for approximately 2 weeks with gentle agitation. Following this, the brains were transferred into kit solution C for 1 week at 40 °C and then sliced in the coronal plane with a cryostat (thickness 150 μ m). The slices were mounted onto subbed microscope slides and stored in darkness for 48 hours. The sections were then rinsed in distilled water twice, for 2 minutes each, and placed in a mixture of kit solution D, E and distilled water (proportioned 1:1:2) for 8 minutes. Finally, sections were cleared in xylene for 4 minutes and coverslipped using DPX mounting medium.

Image analysis

Image stacks from Golgi stained slices were obtained using a DM 6000 Leica microscope with a 100x oil-immersion objective (NA 1.4; Leica, Germany) attached to a Leica digital camera (Leica, DFC350 FX). Image stacks were collected with a 0.2 μ m step size resulting in approximately 30 – 90 images per stack. Microscope and camera settings were adjusted using the Leica Application Suite image acquisition software.

Approximately 20 dendritic images were collected for each brain. The dorsal CA1 region of the hippocampus was targeted, extending from 2.7mm to 4.6mm posterior to bregma²³. Suitable basal dendritic arbors were selected according to the eligibility criteria of previous studies^{19,22}. Segments must be intact and clearly visible (i.e. unobscured by staining artefacts) and isolated from other stained neurons; segments must be fully impregnated by the Golgi stain; segments could not belong to the primary dendritic branch but must be selected from secondary or higher order branches; segments starting and ending extremities were at least 10 μm away from a dendritic branching point or end; the beginning of a segment had to start from a point equidistant between two spines; only one segment per neuron was counted (Figure 1).

ImageJ software (Fiji version 1.51, <https://imagej.net/>) and the Simple Neurite Tracer plugin were used to invert greyscale images and to measure out and crop 20–25 μm dendrite section z-stack images. The images were re-inverted for subsequent processing. Cropped z-stacks were then filtered and sharpened using a custom macro to be finally flattened into a single layer using the in-built temporal colour code hyperstack projection

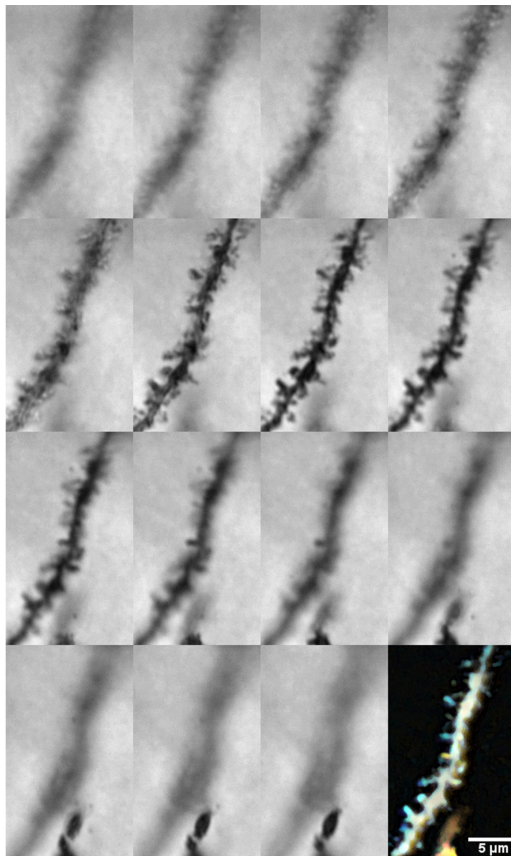


Figure 1. Representative example of an image stack from Golgi-stained dendritic segment of a CA1 basal arbor before and after image processing used for subsequent spine density and clustering analysis.

method. The subsequent processed image represented spines at different depths using different colours. This ensured that spines that may otherwise be hidden were not missed. However, due to the opaque nature of the Golgi stain, spines that are above or below the dendrite with respect to the field of view cannot be visualised. Consistent with previous reports, no attempt was made to count or correct for these inaccessible spines during the counting process¹⁹. Spines were counted manually, and the Cartesian coordinates of identified spines were transformed onto a 1D map of the dendritic branch. During counting, spines with two or more heads (i.e. branched spines) were counted as one spine. Spine density (number of spines / 10 μm dendrite length) and mean nearest neighbour (distance to the nearest spine) for each segment were calculated within ImageJ and imported into RStudio to derive mean case values. In total, 537 CA1 segments were included, from which 6504 spines were counted²⁴.

Statistical analyses

Statistical analyses were carried out using SPSS (version 25, IBM corporation). The threshold for significance was set at $p < 0.05$ unless otherwise specified, i.e. corrected for multiple comparisons. In addition to classical hypothesis testing, the default Bayes factor was calculated to quantify the relative evidence for the null hypothesis (H_0) compared to the alternative hypothesis (H_1). The Bayes Factor (BF10), provides a continuous measure of evidence where a BF10 of 1 indicates that the findings are equally likely under H_0 and H_1 , a BF10 less than 1 indicates support for H_0 over H_1 , and BF10 greater than 1 indicates support for H_1 over H_0 ²⁵. For example, A BF10 of greater than or equal to 3 suggests that the data are 3x more likely under H_1 than H_0 and could be considered ‘substantial’ evidence for H_1 . In comparison, a BF10 of 0.1 suggests the data are 10x more likely under H_0 than H_1 and could be considered ‘substantial’ evidence for H_0 ²⁶. A BF10 range between 1/3 – 3 could be interpreted as ‘anecdotal’ evidence for the H_0 or H_1 ²⁶. Here it is important to note that verbal labels used to categorise different Bayes factors can be useful to facilitate scientific communication, but caution is needed due to the arbitrary nature of these labels and the continuous nature of the Bayes factor²⁶. Default Bayes Factors were calculated using JASP (version 0.11.1).

Results

Due to incomplete staining, only 24 out of 30 cases were suitable for spine density analysis ($n=8$ in each group); only these animals were included in subsequent analyses.

Behaviour

A repeated one-way ANOVA showed a significant improvement in performance across training in the radial-arm maze task, both on the standard ($F(11) = 6.21$, $p < 0.01$) and the rotated variants ($F(5) = 3.54$, $p = 0.04$) (Figure 2)²⁴.

Spine density and clustering

There were 191 sections with 2401 spines in the home-cage control group ($n=8$), 184 sections with 2119 spines in the yoked-control group ($n=8$) and 162 sections with 1984 spines in the spatial

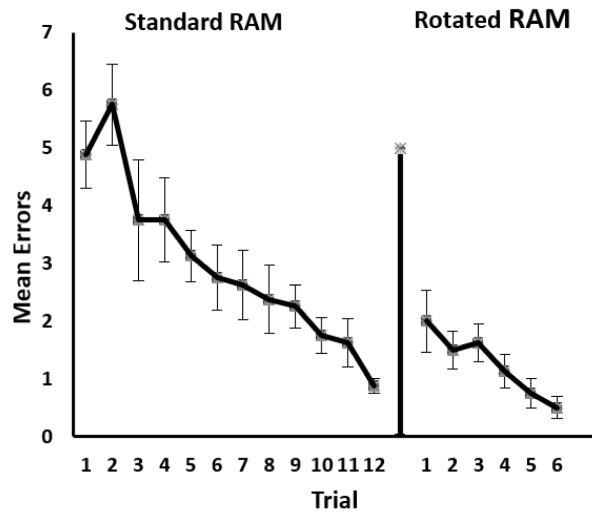


Figure 2. Radial-arm maze (RAM) training. There was a significant reduction in mean number of errors in both the standard and rotated phases of the task. Error bars are \pm the SEM.

memory group ($n=8$)²⁴. One-way between-group ANOVAs found no significant difference between groups for spine density ($F(2) = 1.65$, $p > 0.1$, $BF_{10} = 0.447$) or mean distance to nearest neighbour, i.e., clustering ($F(2) = 0.49$, $p > 0.5$, $BF_{10} = 0.245$; Figure 3). To rule out the possibility that mean nearest neighbour simply reflected spine density, we investigated whether these values co-varied using Pearson's correlation. No relationship was found ($r = -0.064$, $p = 0.79$), indicating that spines were distributed non-randomly. Accounting for spine density by dividing mean nearest neighbour by spine density did not affect the results.

A Bonferroni corrected Pearson's correlation, using an adjusted-alpha level of 0.025, found no significant correlation between errors on the last three trials of the rotated version of the task and spine density ($r = 0.737$, $p = 0.037$) or between errors and mean distance between spines ($r = -0.315$, $p = 0.448$) (Figure 4).

Discussion

The present study failed to find an effect of radial-arm maze training on the density or clustering of spines on the basal arbors of CA1 neurons or their clustering. As such, the present results have not replicated findings from a previous study, which showed increased spine density following radial-arm maze training and a correlation between spine density and behavioural performance^{21,22}. This then raises the question, why the difference across studies?

Hippocampal dendritic spines are temporally dynamic structures and, as such, the time at which they are assessed may be a critical factor in whether or not differences in treatment groups are found. Many behavioural studies do not report the post-training time period that is being assessed, however,

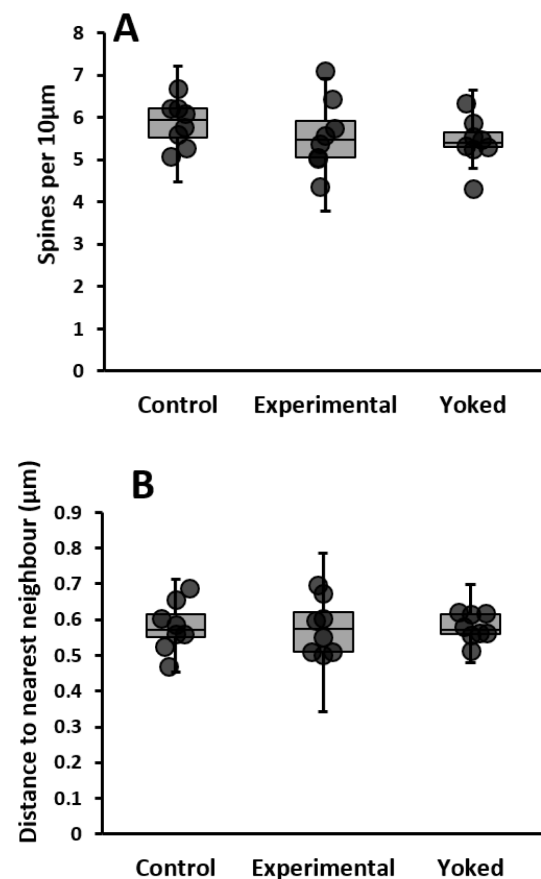


Figure 3. Spine morphology of CA1 basal dendrites for the home-cage control (Control), spatial memory (Experimental) and yoked-control (Yoked) groups. (A) Spine density per 10 μ of basal dendrites in the CA1 region was not significantly different between groups. (B) Mean distance between spines and their nearest neighbour (i.e., clustering) was not significantly different between groups. The central line in each box indicates the median value. The box extends from the first to the third quartile range. The whiskers extend 1.5x the interquartile range. Individual data points are shifted along the x-axis to aid visualisation of overlapping data points.

from the information that is available, behaviourally induced changes in spines have been found during a range of time periods using single time-point techniques. For example, Mahmoud *et al.*²¹ found increased CA1 basal spine density when rats were perfused 6 hours post-training on a radial-arm maze task whilst Harland *et al.*¹⁹ reported increased CA1 basal spines when rats that were previously housed in enriched environments were perfused 24h post-training on a radial-arm maze task.

However, Rusakov *et al.*³ found changes in CA1 spine clustering, but no change in density, 6 days after water-maze training. More detailed information on the time-course of CA1 spine formation and turnover can be acquired from slice-studies. Bourne *et al.*²⁷ showed initial plasticity, including

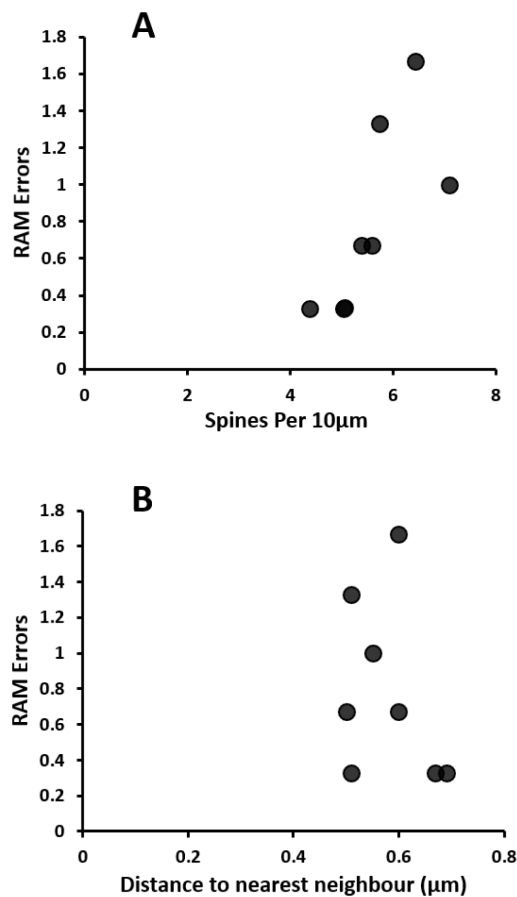


Figure 4. Correlations between spine morphology of CA1 basal dendrites and mean number of errors on the final three trials of the rotated radial-arm maze (RAM) task. (A) Spine density per 10 μ of basal dendrites in the CA1 region was not significantly correlated with mean number of errors. **(B)** Mean distance between spines and their nearest neighbour was not significantly correlated with mean number of errors.

spinogenesis along the dendritic shaft of CA1 neurons, following stimulation that was designed to mimic long-term potentiation. However, at 2 hours post-stimulation there was no overall change in spine density suggesting a redistribution of spines and a balance between loss and gain of spines²⁷. A further *in vivo* study showed CA1 spines to be predominantly impermanent with lifespans of approximately 5–15 days²⁸. In our study, we perfused the animals 90 minutes after their last radial-arm maze session. As such, we should be in a position to capture both immediate post-learning spinogenesis as well as any longer-lasting changes in numbers or clustering from the previous training sessions.

Another possibility is that the stage of learning is a critical factor in whether behavioural-induced structural changes are observed. The hippocampus appears to be particularly important for initial learning of spatial memory tasks²². It is possible that there was increased spine density and clustering

during the early stages of training but this was not maintained for later stages of training. However, other studies have found differences following 10 sessions of training²¹, which is not dissimilar to the 16 sessions of training used in the present study. Additionally, as spine turnover typically occurs over 5–15 days²⁸, we should also be capturing the effects of earlier training sessions. Furthermore, CA1 activity has been shown to correlate with performance on a radial-arm maze task during late-stage training again suggesting that the stage of learning we assessed was not a critical factor¹⁷.

The current study assessed basal CA1 spines but not apical dendrites. This decision was based on previous research where basal CA1 spine density had been shown to increase following spatial memory training (e.g. 19, 29). Data from apical dendrites has yielded mixed results with some studies finding no effect of spatial training (e.g. 19, 29). However Mahmoud *et al.* (2015)²¹, found increased CA1 basal and apical spine density when rats were perfused 6h after the last of 10 radial-arm maze sessions. In comparison, Watman and Holahan (2014)³⁰ found increases in basal but not apical spines when animals were sacrificed two days post-training and increased apical, but not basal, spines when they were sacrificed 29 days post-training. These studies raise the possibility that changes in apical spines may reflect long-term changes and, as such, if they had been assessed in the current study they may have provided information about changes that had occurred during the initial stages of training. However, the expectation based on previous studies is that basal spines should still have been sensitive to the protocol employed in the current study.

It is common practice for studies using optical methods to examine dendritic spines to classify them as thin, mushroom, or stubby according to the spines head and neck diameters³⁰. It has been argued that thin spines are flexible 'learning spines' that may change in size or even dismantle rapidly during learning whereas mushroom spines could be more stable 'memory spines'³¹. Therefore, changes in thin and mushroom spines could reflect changes in new learning and acquired mnemonic information, respectively. Indeed, previous studies have reported changes in the proportion and density of CA1 basal thin and mushroom spines following spatial memory training^{19,21}. However, there is evidence that the typical light microscopy used in the current and previous studies do not have sufficient spatial resolution to properly resolve the distinguishing features of spines. Consistent with this idea, Tønnense *et al.* (2014)³² used super-resolution stimulated emission depletion (STED) imaging and found only a few percent of spines to be stubby. The same data analysed with 2-photon imaging found that spines appearing stubby almost always had short necks when resolved with STED. In the literature, the estimated 20–25% of spines classified as stubby could in fact have short necks that are not readily seen without super-high resolution imaging^{19,21}. Furthermore, there is a large diversity in the appearance of the spine types. It has been asserted that many cannot be classified due to having intermediate characteristics or characteristics that do not fit within the hypothesized categories³³. Therefore, for these reasons the current study did not classify spines into

discrete categories as doing this may not be informative. Instead, future studies could measure spine head and neck diameters, length, volume etc using a continuous spectrum as has been suggested elsewhere^{30,33,34}.

Another difference across studies is the methodology for assessing spines. Mahmoudi *et al.*²¹ used DiOlistic labelling in order to stain cells using a fluorescent dye, which may be more sensitive than the Golgi approach used in the present study³⁵. The Golgi method of staining certainly has limitations, as it only stains a small percentage of the total neurons present and there is still uncertainty as to which neurons are stained and why³⁶. As such, the stained neurons may not be representative³⁷ and they may not be sufficiently capturing cells active during the task³⁸. Nevertheless, other studies have used Golgi-stained tissue to show behaviourally-induced changes in spine density in CA1 neurons^{19,39} and we have also shown lesion-induced changes in spine number and clustering using the same methodology as that used here²².

Conclusions

Together, the present results suggest that spatial learning is not always accompanied by changes in either the density or clustering of dendritic spines on the basal arbor of CA1 pyramidal neurons. As such, there is a need for additional research to determine the conditions under which CA1 spinogenesis contributes to spatial learning and memory. Using longitudinal *in vivo* imaging to track the formation and location of new spines across training⁴⁰ would better enable us to

assess how spine dynamics correlate with on-going behavioural performance.

Data availability

Underlying data

Figshare: Collection holding data and metadata on CA1 dendritic spine density and clustering in rats following training on a radial-arm maze task, <https://doi.org/10.6084/m9.figshare.c.4910244.v1>⁴¹.

This project contains the following underlying data:

- Numerical data for radial-arm maze performance, spine density and spine clustering
- Raw and processed images of Golgi-stained dendritic spines

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

Access to original slides can be provided upon request to Seralynne Vann (corresponding author; vannsd@cardiff.ac.uk).

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Michael G. Stewart 

Department of Life, Health and Chemical Sciences, The Open University, Milton Keynes, UK

This revised manuscript is a very good response to my concerns, meeting each point.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 18 May 2020

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Bruce Harland 

School of Pharmacy, University of Auckland, Auckland, New Zealand

I have no further comments.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Population and single-cell neuronal recording, spatial memory tasks in rodents, neuromorphological analysis, targeted brain lesions and deactivation, treatments for spinal cord injury

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 04 May 2020

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**Bruce Harland** 

School of Pharmacy, University of Auckland, Auckland, New Zealand

Craig *et al.* investigated whether acquisition of a radial-arm maze task in rats induced increases in CA1 basal dendritic spines. Significant improvement was shown by rats in this behavioral task, and a rotated phase of the experiment provided additional evidence that the task was hippocampally dependent. A yoked control group experienced equivalent locomotion in the maze but without acquisition of the task, providing a robust control alongside home-caged controls. A Golgi stain was used to visualize neuronal structure, a technique that provides very good staining of dendritic spines in particular, which is what was quantified in this study. The technique has some drawbacks, for example, the opaque nature of the stain means that spines above and below the dendrite cannot be visualized. Although dye-labelling techniques overcome this issue, to a degree, they usually have their own drawbacks and limitations. Dorsal CA1 basal dendritic spines were analyzed as a measure of experience-dependent neural plasticity.

The methods in this paper were well chosen and executed, and suitable for comparison to other studies. Interestingly, Craig *et al.* did not find increases in CA1 basal spine density or clustering reported by some other studies after spatial task acquisition. Of particular note is the fact that there was no difference between the spatially-trained rats and the home-cage controls. They provide a well thought out and comprehensive discussion of why there may be differences between studies. **These findings are of real interest to the field** and highlight the need for further research into the nature of spine dynamics after spatial learning.

I would like some additional minor clarifications about the methods.

1. The z-distance between images in the image stacks needs to be provided. The example images in Figure 1 suggest that an acceptable 'step size' has been used, but this needs to be given.
2. Some mention needs to be made about whether spines above or below the dendrites (in the optical field of the microscope images) were counted or quantified in some way, and if so, how. If not, is usual to state that no attempt was made to count these hidden spines. As the hidden spines are inaccessible in the same way for all of the different groups, it should not affect the quantification.
3. It would be useful to know the orientation of the tissue 'slices'; coronal, sagittal?
4. Dendritic sections were captured from dorsal CA1, which is a large extent of the hippocampus especially in the rostral-caudal direction. **This is perfectly acceptable**, and is the broad range specified by many other studies. However, the area considered dorsal is not always consistent

between studies. **If possible**, can the authors provide any additional information about the range of CA1 used? For example, were dendritic segments captured from where dorsal CA1 first emerges rostrally in coronal sections all the way back to the caudal limits of the dorsal region? This would be potentially useful information for future studies, for instance if it turns out that there are differences in neural plasticity related to spatial learning within dorsal CA1.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Population and single-cell neuronal recording (including in hippocampus), spatial memory tasks in rodents, neuromorphological analysis, targeted brain lesions and deactivation, treatments for spinal cord injury

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 04 May 2020

Seralynne Vann, Cardiff University, Cardiff, UK

We thank the reviewer for the helpful comments. We have addressed each in turn.

- 1. The z-distance between images in the image stacks needs to be provided. The example images in Figure 1 suggest that an acceptable 'step size' has been used, but this needs to be given.**
We have clarified that a 0.2µm step size was used to collect approximately 30 – 90 images per image stack.
- 2. Some mention needs to be made about whether spines above or below the dendrites (in the optical field of the microscope images) were counted or quantified in some way, and if so, how. If not, is usual to state that no attempt was made to count these hidden spines. As the hidden spines are inaccessible in the same way**

for all of the different groups, it should not affect the quantification.

We have clarified that spines above or below the dendrite with respect to the field of view are not visible with Golgi stain and hence no attempt to count or correct for them was made.

3. It would be useful to know the orientation of the tissue ‘slices’; coronal, sagittal?

We have clarified that coronal slices were used.

4. Dendritic sections were captured from dorsal CA1, which is a large extent of the hippocampus especially in the rostral-caudal direction. This is perfectly acceptable, and is the broad range specified by many other studies. However, the area considered dorsal is not always consistent between studies. If possible, can the authors provide any additional information about the range of CA1 used? For example, were dendritic segments captured from where dorsal CA1 first emerges rostrally in coronal sections all the way back to the caudal limits of the dorsal region? This would be potentially useful information for future studies, for instance if it turns out that there are differences in neural plasticity related to spatial learning within dorsal CA1.

We have clarified that CA1 basal dendritic segments were imaged from the dorsal region of CA1 extending -2.7mm to -4.6mm posterior to bregma according to the atlas of Paxinos and Watson (2006).

References

Paxinos, G., & Watson, C. (2006). *The rat brain in stereotaxic coordinates: hard cover edition*. Elsevier.

Competing Interests: No competing interests were disclosed.

Reviewer Report 15 April 2020

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Michael G. Stewart

Department of Life, Health and Chemical Sciences, The Open University, Milton Keynes, UK

This is an interesting study which reports that there is a lack of change in CA1 dendritic spine density or clustering in adult rats following training on a radial-arm maze task. The study describes a careful analysis of the behavioural testing and the statistical analyses is provided.

The measurement of spines has been made from basal dendrites on Golgi stained section of CA1. While the quantitative analyses are reported in detail there are some issues when comparing to previous studies, in particular that of Mahmoud *et al.* (2015)¹.

1. The present study used Golgi staining which can be capricious and examined only basal dendrites in CA1, whereas that of Mahmoud *et al.* used Dil staining of slices and so ensured that dendrites are more fully impregnated. Moreover, Mahmoud *et al.* examined both apical and basal dendrites.

2. Dendritic spines can be classified as either thin or mushroom and those without a neck are classified as stubby. Mushroom spines are classified on the basis of head diameter. This method is in agreement with well-accepted methods for spine type classification. However, no attempt was made to classify spine types in this study.
3. Spines can be branched or unbranched, it is not clear whether this was examined.
4. No depth analyses appear to have been made for hidden spines which depends on section thickness, and can affect spine counts, it is not clear whether this has been taken into account.
5. Changes after training could affect synapses on spines, increasing spine head diameter, and this might have occurred without spine size changes.

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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 28 Apr 2020

Seralynne Vann, Cardiff University, Cardiff, UK

Comment 1

The present study used Golgi staining which can be capricious and examined only basal

dendrites in CA1, whereas that of Mahmoud *et al.* used Dil staining of slices and so ensured that dendrites are more fully impregnated. Moreover, Mahmoud *et al.* examined both apical and basal dendrites.

We agree that Golgi staining has limitations with regards to the unpredictable nature of staining and had indeed reported this as a limitation of the present study in the Discussion. In our study, dendrites needed to be fully impregnated to be included for analysis. We have now clarified this in the Image Analysis section of the Methods.

Despite the capricious nature of the Golgi method we think this approach is still informative as others have found behaviourally-induced changes in spine density in CA1 (Leuner *et al.*, 2003; Harland *et al.*, 2014) and we have previously shown lesion-induced changes in spine density and clustering in CA1 (Dillingham *et al.*, 2019). This point was also included in the Discussion.

Previous research has indicated that basal CA1 spine density can increase following spatial memory training whereas data from apical dendrites have yielded mixed results. Harland *et al.* (2014) and Moser *et al.* (1997) found basal, but not apical, spines to be sensitive to spatial training. Wartman and Holahan (2014) found increases in basal but not apical spines where animals were sacrificed two days post-training and increased apical, but not basal, spines when they were sacrificed 29 days post-training. This one study raises the possibility that changes in apical spines may reflect long-term changes and, as such, if we had assessed them in our study they may have provided information about changes that had occurred during the initial stages of training. However, the expectation based on previous studies that basal spines should still have been sensitive to our protocol. We have now included this as part of our Discussion.

Comment 2

Dendritic spines can be classified as either thin or mushroom and those without a neck are classified as stubby. Mushroom spines are classified on the basis of head diameter. This method is in agreement with well-accepted methods for spine type classification. However, no attempt was made to classify spine types in this study.

Previous studies using optical microscopes classified spines into the categories of thin, mushroom or stubby, first proposed by Peters and KaisermanAbramof (1970). However, there is evidence that the typical light microscopy used in studies such as ours does not have the sufficient spatial resolution to properly resolve the distinguishing features of spines. Tønnesen *et al.* (2014) used super-resolution stimulated emission depletion (STED) imaging and found only a few percent of spines to be stubby. The same data analysed with 2-photon imaging found that spines appearing stubby almost always in fact had short necks when resolved with STED.

In the literature the estimated 20-25% of spines classified as stubby could in fact have short necks that are not readily seen without super-high resolution imaging (Harland *et al.*, 2014; Mahmoud *et al.*, 2015). Furthermore, there is a large diversity in the appearance of the spine types. It has been asserted that many cannot be classified due to having intermediate characteristics or characteristics that do not fit within the hypothesised categories (Arellano *et al.*, 2007). Therefore, for these reasons we think classifying spines into discrete categories may not be informative. We have added a paragraph to the Discussion covering this important methodological consideration.

Comment 3.

Spines can be branched or unbranched, it is not clear whether this was examined.

Where a clearly branched spine was identified, it was counted just once (i.e. branched spines were not double counted). No further examination of these spines was carried out. This is now clarified in the Methods.

Comment 4

No depth analyses appear to have been made for hidden spines which depends on section thickness, and can affect spine counts, it is not clear whether this has been taken into account.

We thank the Reviewer for highlighting this. We accounted for depth by collecting z-stacks from the bottom through to the top of the dendrite. Our image processing protocol was then able to project these onto a simple plane using the in-built ImageJ temporal colour code hyperstack projection method. The subsequent processed image represents spines at different depths using different colours. This ensures that spines that may otherwise be hidden at different depths are not missed. This is now clearly stated in the Methods section.

Comment 5

Changes after training could affect synapses on spines, increasing spine head diameter, and this might have occurred without spine size changes.

We thank the Reviewer for raising this point. As mentioned in our response to comment 2, classifying spines into discrete categories is problematic. We think the Reviewer's suggestions to measure spine head diameter could be appropriate if measured on a continuous spectrum. This could also be extended to include spine neck sizes as has been suggested by others (Yuste & Majewska, 2001; Arellano *et al.*, 2007). We have now included this as a suggestion that future studies could utilise alongside spine density and clustering.

Readers considering this approach should note that over 75% of spines in the hippocampus are small spines with head diameters of $<0.6\ \mu\text{m}$. These spines are more prone to rapid formation and elimination (Bourne & Harris, 2007; Bourne & Harris, 2011; MacDougall & Fine, 2014). This should be taken into consideration when designing experiments for subsequent analysis of hippocampal spine head and neck sizes.

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Competing Interests: No competing interests were disclosed.